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Antimicrobial, antioxidant activity and phytochemical analysis of an endophytic species of *Nigrospora* isolated from living fossil *Ginkgo biloba*

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Abstract

Nigropsora sp. was isolated as an endophyte from a medicinal plant, *Ginkgo biloba*. Ethyl acetate extract of the fungus was evaluated for antimicrobial and free radical scavenging (DPPH) activity. Ethyl acetate extract was effective against *Escherichia coli* (R-2046), *Klebsiella* sp. (R-2434), *Staphylococcus aureus* (ATCC 6538), *Candida albicans* (NCIM 3471) and *Geotrichum* sp. (NFCCI- 2521) while it did not show activity against *Saccharomyces cerevisiae* (NFCCI-1248), *Nodulisporium* sp. (NFCCI- 2264) and *Syncephalastrum* sp. (NFCCI-2269). The minimum inhibitory concentration (MIC) of ethyl acetate extract evaluated by tube broth dilution method was recoerded as 2.5mg/ml against all test organisms while by agar dilution method it was recorded as 1.25mg/ml against *E. coli*, 0.15625mg/ml against *S. aureus* and 0.3125mg/ml against *Klebsiella* sp. and *C. albicans.* The TLC-bioautography assay, used to screen the plant extracts for antimicrobial activity and localization of the active compounds indicated the presence of a number of inhibitory compounds with activity against tested microorganisms. Phytochemical analysis revealed the presence phenolic compounds and anthraquinones. *Nigrospora* sp. was also examined for *in vitro* antioxidant activity by DPPH radical scavenging assay. The ethyl acetate extract of the fungus showed potent antioxidant activity with IC_{50} value of 9.28µg/ml compared to the IC_{50} value of standard ascorbic acid, 1.74µg/ml.

Keywords – Bioautographic screening – endophyte – *Ginkgo biloba* – MIC – Phytochemical analysis.

Introduction

Endophytic fungi, by definition, are the fungi which spend the whole or part of their lifecycle colonizing inter-and/or intracellularly inside healthy tissues of the host plants, typically without causing apparent symptoms of disease (Azevedo 2007). Endophytes have been found in nearly all plant families examined to date (Arnold 2000). Recently endophytes are viewed as out-standing source of secondary metabolites and bioactive antimicrobial natural products. Endophytic fungi are of biotechnological interest due to their ability to produce antibacterial, antiviral,

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anticancer, antioxidants, antidiabetic and immunosuppressive compounds (Strobel 2003a, Strobel 2003b, Dryfuss 2003) and biological control agents (Clay 1989, Dorworth 1996, Jalgaowala 2010 & Schardl 1999).

Ginkgo is an ancient dioecious plant. At present, only *Ginkgo biloba* L. exists as a living fossil plant. It has been well studied by botanists around the world due to its unique characteristics (Jin 2008). Its fruits and seeds have been used for the treatment of asthma, cough and enuresis (Zimmermann 2002). Since 1990s, the standardized extract of *Ginkgo biloba* leaves has becoming one of the most popular supplements for memory enhancement. The isolation of endophytic fungi from *Ginkgo biloba* has been performed previously and only a few studies have been done on their metabolites (Chen 2007, Qui 2010). Literature reveals that species of *Nigrospora* are the rich source of bioactive secondary metabolites including plant growth-inhibiting nigrosporolide (Harwood 1995) and phomalactone (Kim 2001), phytotoxic and antibacterial nigrosporins (Tanaka 1997), and phytotoxic lactones (Fukushima 1998). It is well established that compounds isolated and characterized from endophytic fungi have potential for use in modern medicine, agriculture and industry. The present study has been designed to investigate the antimicrobial and antioxidant potential of endophytic fungus *Nigrospora* sp. isolated from *Ginkgo biloba*.

Materials and methods

Isolation of endophytic fungi

Nigrospora sp. was isolated as an endophyte from *Ginkgo biloba* obtained from Sikkim region (India) following previously described procedures (Strobel, 2001). Briefly, external tissues of plant parts were fully exposed to 70% ethanol (EtOH, v/v) prior to excision of internal tissues, which were cultured on standard water agar medium (WA) augmented with 50 µg/mL of streptomycin sulphate. Individual fungal colony growing out of plant tissues were then hyphal tipped and transferred on to potato dextrose agar (PDA). After proper growth and fruiting, fungus was identified on the basis of morphological and cultural characteristics.

Fermentation and extraction

Endophytic *Nigropsora* species was grown on PDA at 28ºC for 5-7 days for obtaining young colonies. Three mycelial fragments (5 mm) of endophyte were inoculated into 250 ml Erlenmeyer flasks containing 100 ml of potato dextrose broth (PDB) and incubated at 28ºC for 7-10 days on shaker at 200rpm. The broth cultures were filtered to separate the mycelia from broth. All cell free culture filtrates were extracted three times with equal volume of ethyl acetate (EtOAc) in a separating funnel. The EtOAc phase was collected and the extract was concentrated by evaporation of solvent.

Antimicrobial assay

Antimicrobial activity of Ethyl acetate extract of *Nigrospora* sp. was determined against *Escherichia coli* (R-2046), *Klebsiella* sp. (R-2434), *Staphylococcus aureus* (ATCC 6538) and *Candida albicans* (NCIM 3471) by agar disc diffusion method. *Escherichia coli* (R- 2046) and *Klebsiella* sp. (R- 2434) were obtained from Ruby Hall clinic, Pune. *Staphylococcus aureus* (ATCC 6538) was procured from American Type Culture Collection, and *Candida albicans* (NCIM 3471) was procured from National Collection of Industrial Microorganisms, National Chemical Laboratory (NCL), Pune. The inoculums of test organisms were standardized so as to obtain 1.5×10^8 CFU/ml. The discs of extract were prepared so that the final concentration on disc is 25µg, 50 µg, 100 µg, 200 µg, 400 µg and 800 µg. Streptomycin sulphate (30 µg) was used as positive control and discs of ethyl acetate were used as negative control.

Antifungal activity of ethyl acetate extract was checked by agar well diffusion method against *Saccharomyces cerevisiae* (NFCCI-1248), *Nodulisporium* sp. (NFCCI- 2264), *Syncephalastrum* sp. (NFCCI-2269), and *Geotrichum* sp. (NFCCI- 2521). All these fungal cultures

were obtained from National Fungal Culture Collection of India, Agharkar Research Institute, Pune. The inoculums of these fungi were standardized so as to obtain spore density of 2×10^6 spores/ml. Crude sample was dissolved in ethyl acetate to prepare stock solution of 4mg/ml and it was inoculated so that the final concentration in well is 25µg, 100 µg and 500 µg per well. Nystatin (30 µg) was used as positive control. Well containing only ethyl acetate was used as negative control. Each test was performed in triplicates.

Determination of minimum inhibitory concentration (MIC)

Determination of MIC by broth dilution method

In broth dilution method, the extract was prepared by two-fold serial dilution using distilled water to obtain concentrations of 5, 2.5, 1.25, 0.625, 0.3125, 0.15, 0.078 and 0.039 mg/ml. Equal volume (2-ml) of extract and Mueller – Hinton broth (MHB) were mixed. 2-ml of standardized inoculum (1.5×10^8 CFU/ml) was added to each of the test tubes containing extract. The inoculated tubes were incubated aerobically at 37^0C for 24 hours. Tubes containing broth and extracts without inoculum served as positive control while tubes containing broth and inoculum served as negative control. The tubes were observed after 24 hours of incubation to determine MIC.

Determination of MIC by agar dilution method

In agar dilution method, stock solutions of extract were prepared by two-fold serial dilutions to obtain concentrations of 50, 25, 12.5, 6.25, 3.125, 1.5, 0.78 and 0.39 mg/ml. The 1-ml of the each extracts was added to 9 ml of sterile molten MHA to obtain final concentrations of 5, 2.5, 1.25, 0.625, 0.3125, 0.15, 0.078 and 0.039 mg/ml and mixture was poured in Petri-plates. After solidification, 0.1ml of standardized inoculum of test culture was inoculated on each plate. These plates were then incubated at 37^0C for 24hrs.

Bioautographic antimicrobial screening

A simple, direct bioautographic assay (Hamburger and Cordell, 1987) on TLC plates was used for screening of compounds with activity against select microorganisms, *Escherichia coli* (R-2046), *Staphylococcus aureus* (ATCC 6538), *Klebsiella* sp. (R-2434) and *Candida albicans* (NCIM 3471) with slight modifications. About 100 μl extract was applied to the TLC plate and developed in a Chloroform ($CHCl_3$): Methanol (MeOH) (10:1 v/v) mobile phase. The developed chromatograms were dried for complete removal of the remaining solvents, and spotted TLC plates were placed upside down on MHA plates inoculated with test organisms. The inoculated plates were incubated at 37^0 C for 24 hours. Antimicrobial compounds were recorded and identified as clear zone of inhibition.

1, 1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity

The DPPH radical scavenging assay was performed as described by Miliauskas et al. (2004) with slight modifications. In brief, 2-ml of DPPH solution (0.06mM) was added to 1-ml of test sample of concentrations (18.75, 37.5, 75, 150 and 300 µg/ml) leading to the final concentration of 6.25, 12.5, 25, 50, and 100 µg/ml. After 24 hours reaction, absorbance was read at 517 nm. Ascorbic acid was used as control. The scavenging ability on DPPH radicals was calculated using the following equation:

$$\% \text{ Radical scavenging activity} = \left(1 - \frac{A_i - A_j}{A_c}\right) \times 100$$

Where Ac is the absorbance of DPPH without extracts, Aj is the absorbance of extracts corresponding to each concentration, and Ai is the absorbance of DPPH with extracts. The antioxidant activity of each sample was expressed in terms of IC_{50} value; concentration (µg/ml)

required to inhibit DPPH radical formation by 50% and was calculated from the graph after plotting inhibition percentage against extract concentration.

Phytochemical screening

EtOAc extract of *Nigrospora* sp. was evaluated for its phytochemical constituents using the procedures of Trease (1989), Sofowora (1993) and Kokate (1995). Alkaloids, carbohydrates, proteins and amino acids, phytosterols, phenolic compounds, flavonoids and anthraquinones were qualitatively analyzed.

Results

Cultivation of the different tissues of *Ginkgo biloba* has led to the isolation of different endophytic fungi. They were successfully identified as: *Colletotrichum gloeosporoides*, *Pestalotiopsis* sp., *Phyllosticta* sp., *Nigrospora* sp. and *Xyalaria* sp. These endophytic fungi were screened for antimicrobial activity by agar well diffusion method against selected pathogenic bacteria and fungi. Among these, an endophytic fungus *Nigropsora* sp. showed strong inhibitory activity in plate assay against growth of pathogens and hence it was selected for further studies.

Nigrospora sp. was found to produce woolly colonies on PDA at 28^0C. Colonies were white at first with small, shinining black conidia which were easily visible under a low power dissecting microscope. Later on, colonies became blackish. Red colour soluble pigment in PDA was also noticed from this endophyte (Fig. 1–A). Under microscope septate hyaline hyphae, hyaline and pigmented conidiophores, and conidia were visualized. Conidiogenous cells recorded were monoblastic, solitary, ampuliform, bear single conidium at the apex. Conidia were black, shining, spherical or ellipsoidal having mean diameter of 14.59µm, slightly flattened and 0-septate (Fig. 1–B). Based on above mentioned morphological taxonomic characteristics, the fungal isolate was identified as species of *Nigropsora* (Ellis 1971).

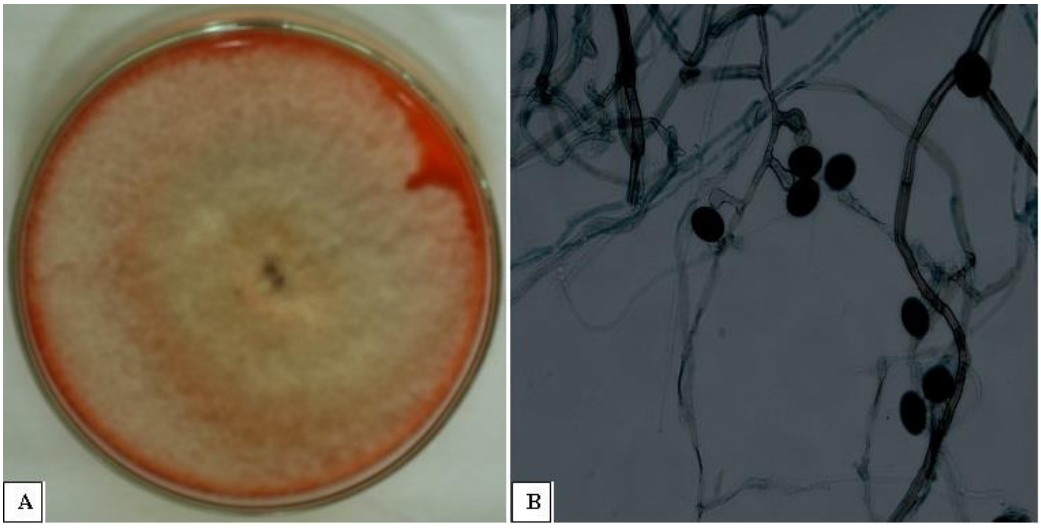

Fig. 1 – *Nigrospora* sp. isolated from host plant *Ginkgo biloba*. A Culture morphology of endophytic *Nigrospora* sp. grown on PDA, B microscopic observations

In present study, antimicrobial activity of red coloured ethyl acetate extract of *Nigropsora* sp. against *Escherichia coli* (R-2046), *Klebsiella* sp. (R-2434), *Staphylococcus aureus* (ATCC 6538) and *Candida albicans* (NCIM 3471) was determined by agar disc diffusion method. The antimicrobial activity of extract showed little variation and excellent reproducibility of zone of inhibition for all selected pathogens within 25-800 µg/ml concentration range. Evolution of zone of inhibition was observed at lowest concentration 25µg/ml where the mean diameter of zone of inhibition was 10 mm against *E. coli*, 13mm against *S. aureus*, 14mm against *Klebsiella* sp. and

10.66mm against *C. albicans*. The results showed that increase in concentration of extract increased the zone of inhibition. Among all selected four microorganisms, different size of zone of inhibition was exhibited at 25-100 µg/ml in increasing order 10-21.66mm against *E. coli*, 14-19mm against *Klebsiella* sp., and 10.66-20.33mm against *C. albicans* while against *S. aureus* similar zone of inhibition of 13mm was obtained at concentration 25-100 µg/ml and then increased up to 19.33mm at concentration of 800 µg/ml (Table 1). This antimicrobial activity by disc diffusion method was represented in Fig. 2.

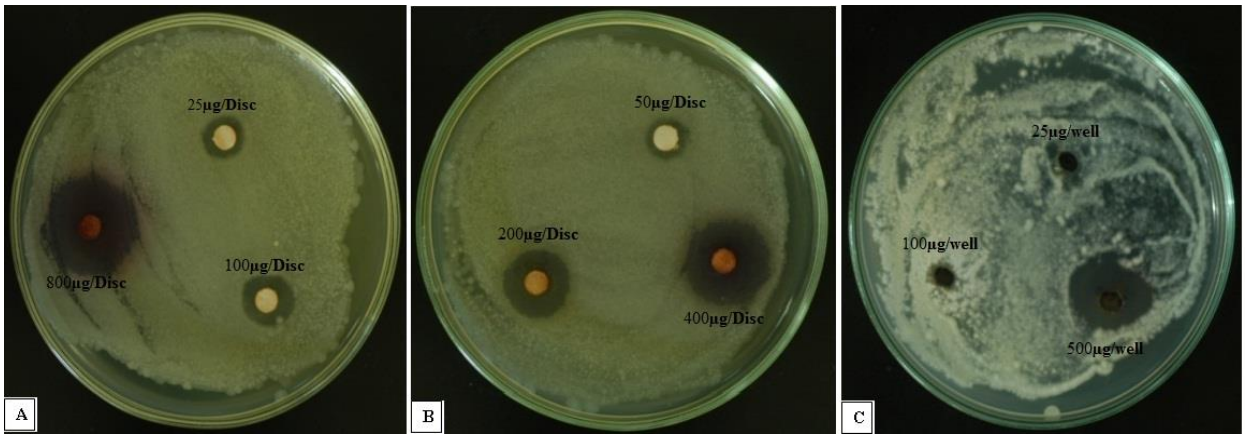

Fig. 2 – Antimicrobial activity of ethyl acetate extract of *Nigrospora* sp. A–B Antibacterial activity against *E. coli*, C Antifungal activity against *Geotrichum* sp.

Table 1 Level of zone of inhibition of ethyl acetate extract against selected microorganisms by disc diffusion method

Test organism	Diameter of zone of inhibition (mm)					
	25 µg/disc	50 µg/ disc	100 µg/ disc	200 µg/ disc	400 µg/ disc	800 µg/ disc
E. coli	10	12	13.66	16.33	20.33	21.66
S. aureus	13	13	13	15.33	17.66	19.33
Klebsiella sp.	14	14.33	15.66	16.33	18	19
C. albicans	10.66	14.33	14.33	16.66	18.66	20.33

In present study activity of ethyl acetate extract of *Nigrospora* sp. against *Saccharomyces cerevisiae* (NFCCI-1248), *Nodulisporium* sp. (NFCCI- 2264), *Syncephalastrum* sp. (NFCCI-2269) and *Geotrichum* sp. (NFCCI- 2521) was investigated by agar well diffusion method at concentration of 25µg, 100 µg and 500 µg per well. Extract did not show activity against *Saccharomyces cerevisiae*, *Nodulisporium* sp., *Syncephalastrum* sp. while it showed activity only against *Geotrichum* sp. Evolution of zone of inhibition was observed against *Geotrichum* sp. at concentration of 500 µg/well while no zone was observed at concentration of 25µg and 100 µg per well (Fig 2-C). Antifungal activity of extract was shown in Table 2.

Table 2 Antifungal activity of ethyl acatate extract of endophytic fungus *Nigrospora* sp.

Test Fungi	Diameter of zone of inhibition (mm)		
	25 µg/well	100 µg/well	500 µg/well
Saccharomyces cerevisiae (NFCCI-1248)	No zone	No zone	No zone
Nodulisporium sp. (NFCCI- 2264)	No zone	No zone	No zone
Syncephalastrum sp. (NFCCI-2269)	No zone	No zone	No zone
Geotrichum sp. (NFCCI- 2521)	No zone	No zone	19

The antimicrobial potential of ethyl acetate extract of endophytic fungal culture was investigated using the minimal inhibitory concentration (MIC) assay against *Escherichia coli* (R-2046), *Klebsiella* sp. (R-2434), *Staphylococcus aureus* (ATCC 6538) and *Candida albicans* (NCIM 3471) using broth and agar dilution method over the range 0.039-5mg/ml. The MICs of extract of *Nigrospora* sp. are shown in the Table 3.

Table 3 MIC values of EtOAc extract of endophytic fungus *Nigrospora* sp.

Microoganism	**MIC (mg/ml)**	
	Broth dilution method	**Agar dilution method**
E. coli	2.5	1.25
S. aureus	2.5	0.15625
Klebsiella sp.	2.5	0.3125
C. albicans	2.5	0.3125

MIC was defined as the lowest concentration of *Nigrospora* sp. extract at which no colony or no growth was observed after incubation. By broth dilution method, ethyl acetate extract of endophytic fungus showed antimicrobial activity against all test microorganisms with MIC of 2.5mg/ml, while by agar dilution method it showed antimicrobial activity with MIC of 1.25mg/ml against *E. coli,* 0.15625mg/ml against *S. aureus* and with MIC of 0.3125mg/ml against *Klebsiella* sp. and *C. albicans.*

Bioautography, as a method to localise antimicrobial activity on a chromatogram has found widespread application in the search of new antibiotics. This assay has the advantage of being quick, easy to perform and relatively cheap, requiring no sophisticated infrastructure, only requiring a small amount of the test compound, with high sample throughput and the results are easy to interpret. Metabolites with antimicrobial activity were identified as clear zone on MHA plate containing bacterial inoculum and TLC plate developed in solvent system. One zone of inhibition (R_f =0.6) resulting from compound in extract found to have inhibitory activity against *Escherichia coli*, *Klebsiella* sp., *Staphylococcus aureus* and *Candida albicans*. The TLC bioautography of extract against *E.coli* is shown in Fig. 3.

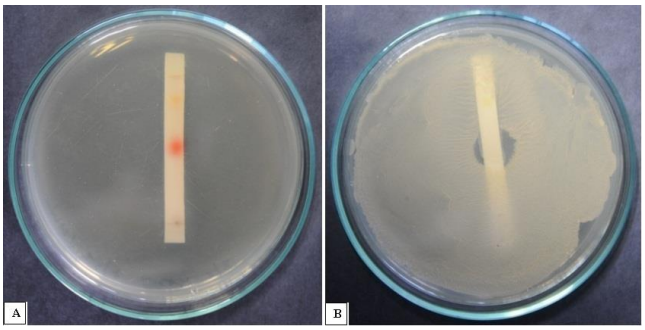

Fig. 3 – TLC bioautography of EtOAc extract of *Nigrospora* sp. against *E.*coli. A Before incubation, B after incubation of 24 hrs at 37^0C

To determine the effects of endophytic fungal extract of *Nigrospora* sp. on *in vitro* antioxidant activity, the DPPH scavenging rate was studied. The DPPH radical contains an old electron, which is accountable for the absorbance at 517 nm and also for a visible deep purple color. DPPH is decolorized when it accepts an electron donated by an antioxidant compound, which can be quantitatively measured from the changes in absorbance. The antioxidant activity of each sample was expressed in terms of IC_{50} value which was calculated from the graph after plotting inhibition percentage against extract concentration. The EtOAc extract of *Nigrospora* sp. exhibited antioxidant activity with IC_{50} value of 9.28µg/ml compared to the IC_{50} value of ascorbic acid 1.74µg/ml (Table 4).

Table 4 DPPH radical scavenging activities of EtOAc extract of endophytic fungus *Nigropsora* sp. and ascorbic acid.

Test	DPPH radical scavenging activity (%)					IC_{50}
	6.25(µg/ml)	12.5(µg/ml)	25(µg/ml)	50(µg/ml)	100(µg/ml)	(µg/ml)
EtOAc extract	39.93	87.92	100.69	95.04	100.26	9.28
Standard (Ascorbic acid)	97.3	98.93	98.87	99.06	99.12	1.74

The results showed that increase in concentration ascorbic acid increased % radical scavenging activity. But for fungal extract after repetitive experiments for antioxidant activity, it is observed that % radical scvenging activity of extract at concentration 25µg/ml (100%) is more than activity at concentration 50µg/ml (95.54%) and almost equal to activity at concentration 100 µg/ml (100 %). This might be because of interference of absorbance of crude extract. At concentration of 25µg/ml, absorbance of red colored extract (A_j) was always higher than absorbance of yellow color produced after reduction of DPPH by extract (A_i). The extract showed an inhibition in free radical production, almost an equipotent effect with standard antioxidant ascorbic acid.

Phytochemical analysis of EtOAc extract of *Nigropsora* sp. showed presence of phenolic compounds and anthraquinones. These phytochemicals may be responsible for antimicrobial and antioxidant activity evaluated in present study.

Discussion

During this study, a review of literature has been conducted for the diversity of endophytic fungi associated with the living fossil *Ginkgo biloba*. A total of five fungal endophytes were isolated and cultured in the laboratory. Morphological investigations, using both macroscopic and microscopic features, have resulted in the identification of five fungal species: *Colletotrichum gloeosporoides, Pestalotiopsis* sp., *Phyllosticta* sp., *Nigrospora* sp. and *Xyalaria* sp. Overall, these preliminary results suggest that *Ginkgo biloba* hosts many species of endophytic fungi.

The need for new antimicrobial agents, in general, comes from the increasing rates of resistance to existing antibiotics. In this study, an initial assessment was performed for the antibacterial and antifungal activity of the isolated endophytic fungi. The EtOAc extract of endophytic fungus *Nigrospora* sp. showed potential antimicrobial activity. Literature reveals that endophyte belonging to the genus *Nigrospora* have been a rich source of bioactive secondary metabolites, such as plant growth-inhibiting nigrosporolide (Harwood, 1995) and phomalactone (Kim, 2001), phytotoxic and antibacterial nigrosporins (Tanaka, 1997), and phytotoxic lactones (Fukushima, 1998)**.**

From our study, it has been concluded that EtOAc extract from endophytic fungus *Nigrospora* sp. has potential antimicrobial activity against pathogenic bacteria and yeast as compared to that of against fungi. In our study, some of the fungal strains did not respond to extract, might be due to masking of antimicrobial activity by the presence of some inhibitory compounds or factors in the extract or synergism by the presence of some compounds or factors in the extract. This might also be due to range of concentration of extract tested and test pathogenic fungi selected for activity. Antifungal activity against selected fungi might be observed at concentration more than 500 µg or also against pathogenic fungi other than the test fungi used in this study or the extract might yield antifungal activity once it undergoes some purification. Zhao (2012) reported antifungal activity of purified bioactive secondary metabolites from *Nigrospora* sp. LLGLM003, an endophytic fungus of the medicinal plant *Moringa oleifera* Lam.

From study of MIC, it has been observed that the extract was more active against Gram-positive bacteria, *S. aureus* than Gram-negative bacteria *E. coli* and *Klebsiella* sp. In present investigation MICs of extract obtained by broth dilution method recorded higher than that of MICs obtained by agar dilution. This might be due to interference of turbidity of extract in turbidity of

bacterial growth in broth dilution method. Similarly, Trisuwan (2008) reported MIC value of purified compounds from marine derived *Nigrospora* sp. The applied EtOAc of *Nigrospora* sp. had antimicrobial activity by bioautographic TLC assay.

The DPPH radical scavenging activity of extract of *Nigropsora* sp. exhibited antioxidant activity with IC_{50} value of 9.28µg/ml in present study. Even though the DPPH scavenging aptitude of the extracts was found to be lower at concentrations of 6.25-50µg/ml than that of the activity of commercial antioxidant, ascorbic acid at same concentrations, it still reached 100% inhibition at 100µg/ml concentration. This study shows that the extract have proton donating ability and could serve as free radical inhibitors/ scavenging, acting as primary antioxidant. Rukachaisirikul (2010) has also evaluated antioxidant activity of purified compounds from *Nigrospora* sp. This antimicrobial and antioxidant activity of extract of *Nigrospora* sp. is might be due to presence of phenolic compounds or anthraquinones confirmed by phytochemical analysis. Thus, this study suggests that the *Nigrospora* sp. has highlighted the potentiality as natural antimicrobials and antioxidants.

Conclusion

The present study concludes that the presence of bioactive compound in the extract exhibited antimicrobial and antioxidant activity in *Nigrospora* sp. Furthermore, active crude extracts are being subjected for the isolation and identification of active antimicrobial compounds which may provide a better source for developing new therapeutic agents. These obtained antibacterial and antioxidant compounds would be then required to be evaluated against wider range of bacterial strains as well as *in vivo*, and tested for their safety and efficacy as therapeutic principles against infectious disease. Moreover, attempts may be required to use analytical chemistry procedures, in order to isolate and identify the bioactive principle compounds responsible for the antioxidant activity reported here.

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